Cancer Vaccine and Therapy

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation with distinction in Molecular Genetics in the undergraduate colleges of The Ohio State University

by

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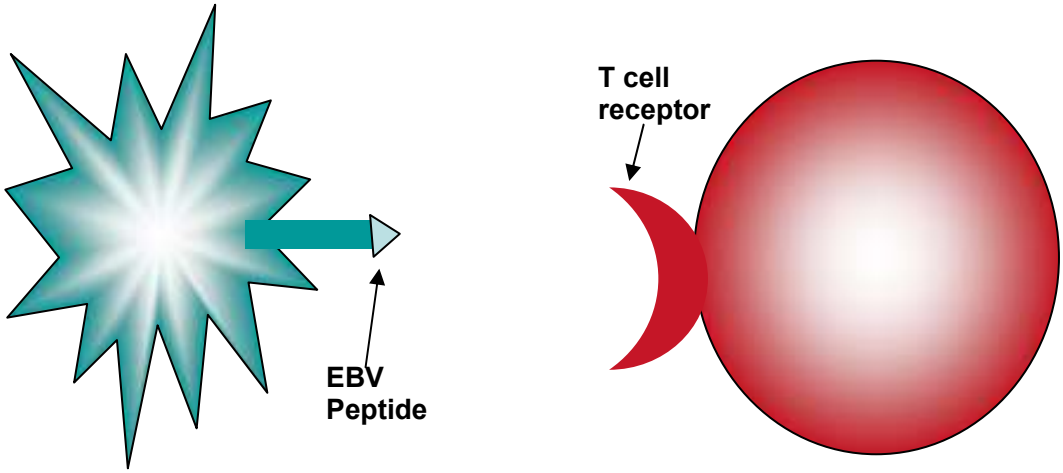
Background

Research has demonstrated a relationship between cancer and the human immune system. Certain cancers have a higher incidence in patients with compromised immune systems. An example occurs in organ transplant patients who are taking immunosuppressive drugs and go on to develop post-transplant lymphoproliferative disease (PTLD). PTLD complicates between 2% and 20% of solid organ transplants performed annually in the United States and is associated with the Epstein-Barr Virus (EBV) infection in 95% of the cases.

Epstein-Barr Virus

EBV is the best known and most widely studied herpes virus and was also the first virus implicated in a human cancer. EBV is associated with other cancers, such as Burkitt's lymphoma, Hodgkin's disease, and Kaposi's sarcoma. A unique feature of the virus is that it is present, although dormant, for the lifetime of 90% of immunocompetent adults. EBV infection can arise during childhood and adolescence and occurs as infectious mononucleosis ("mono") in 50% of these humans. Healthy immune systems create T cells (immune cells in the blood) that are capable of controlling the EBV infection. T cells become specific for EBV by recognizing the peptide from EBV proteins on the surface of an antigen presenting cell (APC) (reference figure 1). Healthy humans are able to activate CD8+ cytotoxic T lymphocytes (CTLs) to recognize and eliminate virus-infected cells. However, humans with compromised immune systems are unable to produce sufficient T cells to control the infection [1].



Post-Transplant Lymphoproliferative Disease

Post-Transplant Lymphoproliferative Disease (PTLD) is typically linked to EBV infection and has many features of immune system malignancy. Recently, the incidence of PTLD has been on the rise and it has emerged as a significant complication of solid organ and cell transplantation [2]. It is characterized by uncontrolled proliferation of B cells when post-transplant patients are undergoing immunosuppresion. PTLD presents significant challenges for physicians because it is difficult to predict and has high morbidity and mortality rates.

Patients undergoing organ transplants have compromised immune systems due to the immunosuppressive medications they are prescribed in order to avoid organ-rejection. Because of this, these patients have impaired immune function and lack the capabilities to fight off EBV and other typically dormant viruses. Therefore, these patients become more likely than immunocompetent humans to develop PTLD.

There are currently a variety of methods that physicians have been using to attempt to treat PTLD. Researchers and clinicians are in need of a treatment method

that is able to both treat the tumor and maintain an immunosuppressed state to preserve the organ donation [3, 4, 5]. This has been attempted through the reduction of immunosuppressive medications, radiotherapy, chemotherapy, cell therapy, antiviral therapy, and cytokine therapy.

In a recent study, a reduced amount of immunosuppressive drugs was administered in order to reactivate the immune system to attack the EBV-associated tumor [6]. This did, in fact, reduce the tumor in 9 of 11 patients, but led to organ rejection in 5 cases. This study, for the first time, showed an increase in the CD3+CD8+ T cells in patients that went into remission. Another recent study used HLA tetramers complexed with EBV immunodominant peptides and found that a subset of CD3+CD8+ T cells were EBV specific [1]. They also found in a severe combined immune deficient (SCID) mouse model system, as well as in patients, that the expansion of an EBV protein, BZLF1 (RAK) specific CD8+ T cells, correlated with the prevention of PTLD after a combined cytokine treatment. These findings opened a door in the research of human cellular subsets in mediating this protective effect.

A vaccine approach to increase the quantity of T cells is a possible treatment method that could be beneficial to many patients. Since PTLD results in mortality in 50-70% of patients, a vaccine to reduce the frequency of infection could be especially advantageous to organ transplant patients.

We hypothesized that a vaccine of BZLF1 protein transduced monocyte-derived antigen presenting cells (dendritic cells) will stimulate the expansion of Epstein-Barr Virus (EBV) BZLF1-specific cytotoxic CD8+ T lymphocytes (CTLs).

In order to complete this, we developed two specific aims:

Aim 1 – Goal A: Synthesize a BZLF1/GST fusion protein using the Glutathione S-transferase (GST) gene fusion system from prokaryotic cells.

Goal B: Synthesize a BZLF1/ V5-His fusion protein from mammalian cells.

Aim 2: Determine if monocyte-derived DCs pulsed with BZLF1 fusion proteins stimulate expansion of EBV BZLF1-specific CTLs.

Methods

Preparation of the Target Protein

The preparation and purification of BZLF1, an EBV viral protein, was accomplished using standard protocols provided by Novagen and Invitrogen Corporation for Goals A and B respectively (reference *flow sheet 1*). For Goal A, EBV BZLF1 cDNA was cloned into a prokaryotic expression vector, pET, obtained from Abgent Inc.

For Goal B, a mammalian expression vector, pUB6/V5-His, was prepared by digestion with restriction enzymes (EcoR1 and BamH1) and dephosphorylation with calf intestinal alkaline phosphatase. The vector was then gel purified. Next, the insert DNA containing a BZLF1 coding sequence was prepared. This was accomplished by plasmid prep and PCR (in order to amplify the BZLF1 coding sequence). This was followed by a restriction digest with (EcoR1 and BamH1) and gel purification. The DNA insert was then ligated into the pUB6/V5-His vector.

For both Goals A and B, each insert/vector pair was transformed into the DH5α strain of E. *coli* cells. These cells served as an expression host. Finally, the expression of BZLF1 was analyzed. A positive clone was sequenced and the expression of BZLF1 protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Western blot. The positive clones of generated stable Chinese Hamster Ovarian (CHO) cell lines were expanded in order to generate a large quantity and cell pellets were frozen at -80˚C until purification took place.

Purification of the Target Protein

For Goal A, the BZLF1 was purified following a standard protocol (GST purification kit from Amersham Bioscience).

For goal B, the BZLF1 was purified following standard protocol provided by Invitrogen's ProBond Purification System. The cell lysate was prepared under native conditions and added to a prepared His affinity column. The lysate was allowed to bind to the resin, then the column was washed, and the protein was eluted. The protein concentration was measured at UV280 and the eluted fractions were stored at 4˚C. Samples of the lysate and wash supernatants were also saved for SDS-PAGE analysis.

Functional test of the Purified Target Protein

Next, functional testing of the purified BZLF1 protein was conducted (reference *flow sheet* 2). First, monocyte-derived dendritic cells transduced with BZLF1 protein were generated by culturing hPBMC for 3 days, with cytokine GM-CSF and IFN-ɑ in the presence of EBV BZLF1 protein. BSA (Bovine Serum Proteins) served as a negative

control. The presence of the BZLF1 protein in the dendritic cells was determined by immunohistochemistry staining. Next, dendritic cells transduced with BZLF1 were co-cultured with hPBMC from the same donor (autologous). Following the culture, BZLF1-specific T cells were expanded. The activation and cytotoxicity of the generated BZLF1-specific T cells was also determined. The activation of CD8+ T cells was determined by IFN-γ secretion assayed by an IFN-γ-Elispot or by intracellular IFN-γ staining. The cytotoxicity of CD8+ BZLF1-specific T cells was determined by co-cultured CD8+ T cells with a ^{51}Cr-labeled B cell line derived from the same donor. The cytotoxicity of T cells was then calculated based on the percentage of ^{51}Cr released.

Preparation and Purification of the Target Protein

Flow Sheet 1 (Goal B specifically)

Process	**Detail**
Prepare pUB6/V5-His Vector	1. Digest with restriction enzyme and dephosphorylate 2. Gel purify
↓	
Prepare Insert DNA (BZLF-1 coding sequence)	1. Plasmid prep and PCR 2. Restriction digest 3. Gel purify
↓	
Clone Insert into pUB6/V5-His Vector	1. Ligate or anneal insert with pUB6/V5-His vector 2. Transform into non-expression host 3. Identify positive clones; colony PCR, prepare plasmid DNA, verify reading frame by sequencing, or *in vitro* transcription/translation
↓	
Transform into Expression Host (E. *coli*)	1. Transform DH5α E. *coli* cells
↓	
Analysis of BZLF1 Expression And Generate CHO stable cell lines	1. Detect target protein by SDS-PAGE, Western blot
↓	
Scale-up	1. Scale up culture 2. Prepare extract 3. His Affinity column purification
↓	
Purify Target Protein	

Test of the Purified Target Protein

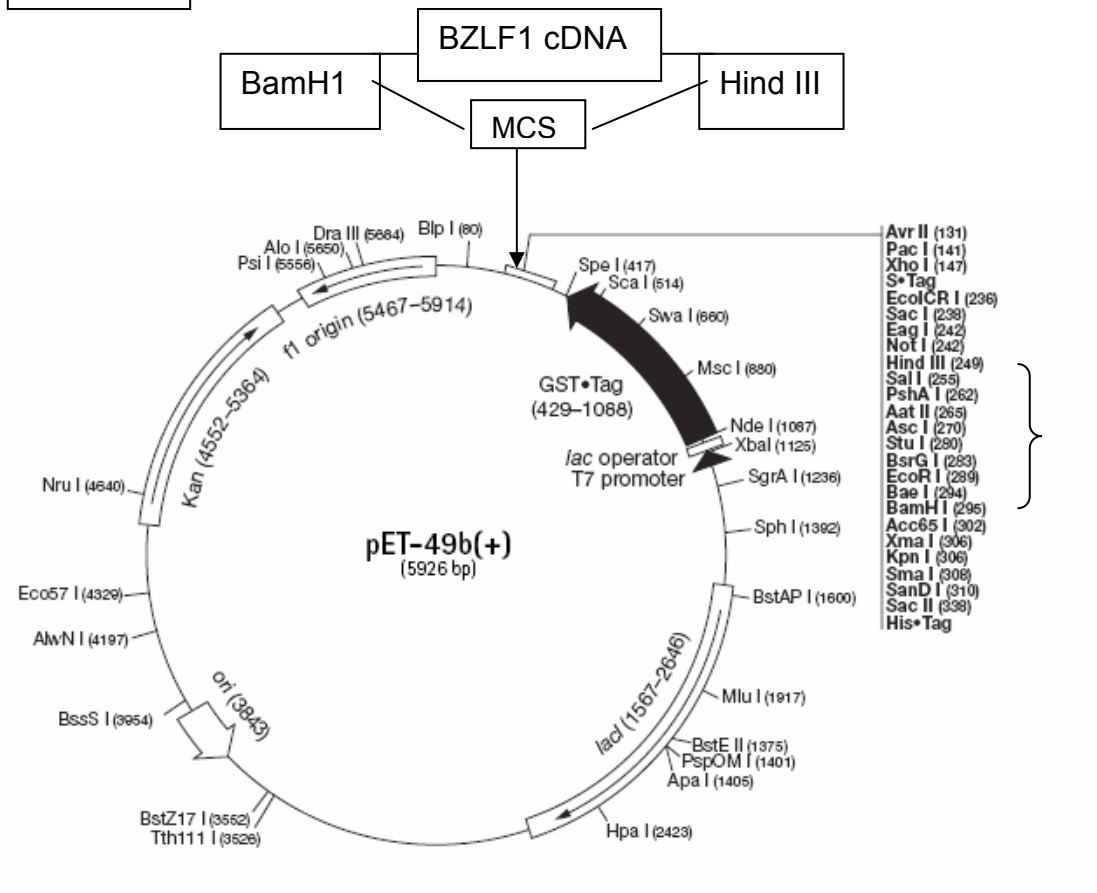
Flow Sheet 2

Process	**Detail**
Generation of monocyte-derived DCs transduced with BZLF1 protein	1. Culture hPBMC short term (5 days) with GM-CSF in the presence of EBV BZLF1 protein. 2. The presence of BZLF1 protein in DCs will be determined by an immunohistochemistry staining. 3. Co-culture BZLF1+ DCs with hPBMC from same donor
↓	
Expansion of BZLF1 specific T cells	4. The activation of CD8+ T cells can be determined by IFN gamma secretion assayed by a gamma IFN-Elispot.
↓	
Determination of activation and cytotoxicity of generated BZLF1 specific T cells	5. The cytotoxicity of CD8+ BZLF1 specific T cells will be determined by co-cultured CD8+ T cells with a ^{51}Cr labeled same donor derived B cell line. The cytotoxicity of the T cells will be calculated based on the percentage of ^{51}Cr released.

Results

The intention of Aim 1-Goal B was to synthesize EBV BZLF1-GST fusion protein *in vitro*. In order to generate a BZLF1 protein fused with the GST tag, EBV BZLF1 full length cDNA was amplified by Polymerase Chain Reaction (PCR) and cloned into a vector (pET vector, *figure 1*). The pET vector was prepared by digestion with restriction enzymes (BamHI and HindIII) and dephosphorylation with calf intestinal alkaline phosphatase. The vector was then gel purified. The cloning was successful as evidenced by many more colonies produced from ligation in the presence of the insert compared with the negative control. The positive clones were identified by colony PCR and restriction enzyme digestion.

Figure 1

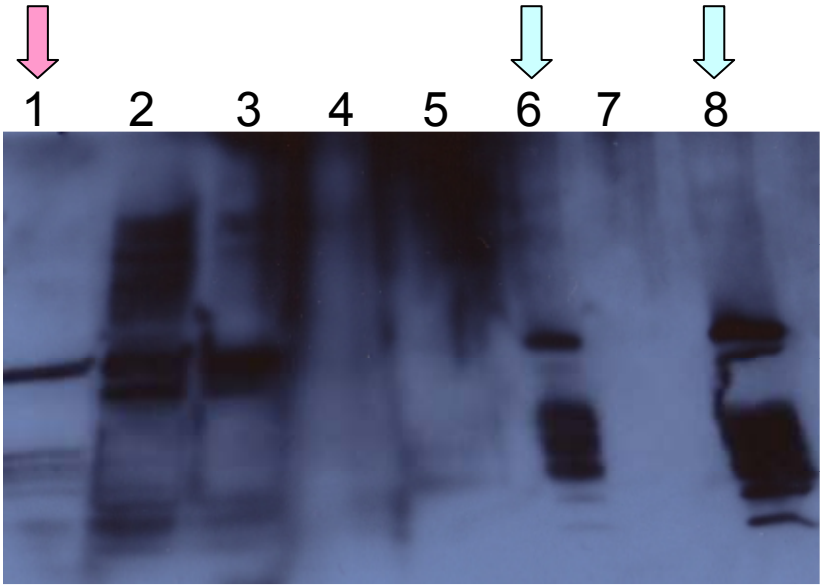


A pET vector was prepared and BZLF1 cDNA was inserted.

The BZLF1 was then expressed in appropriate competent cells (BL21). At this point, the expression of the BZLF1 protein was optimized by analyzing plasmid stability and protein solubility. The BZLF1 protein expression was induced with IPTG, detected, and purified. The purity of the isolated EBV BZLF1 protein was determined by a Western blot using a monoclonal antibody against BZLF1 (*figure 2*). The Western blot shows that the IPTG induced BZLF1 protein expression, as shown in figure 2, lanes 6 and 8 compared with lane 1.

Figure 2

Western Blot of IPTG Induction



Lane 1: No IPTG
Lane 2: IPTG without purification
Lane 3: Lysate before sonication
Lane 4: Insoluble pellet
Lane 5: N/A
Lane 6: 10/50 ul IPTG
Lane 7: N/A
Lane 8: 20/50 ul IPTG

IPTG induced BZLF1 protein expansion (lanes 6 & 8).

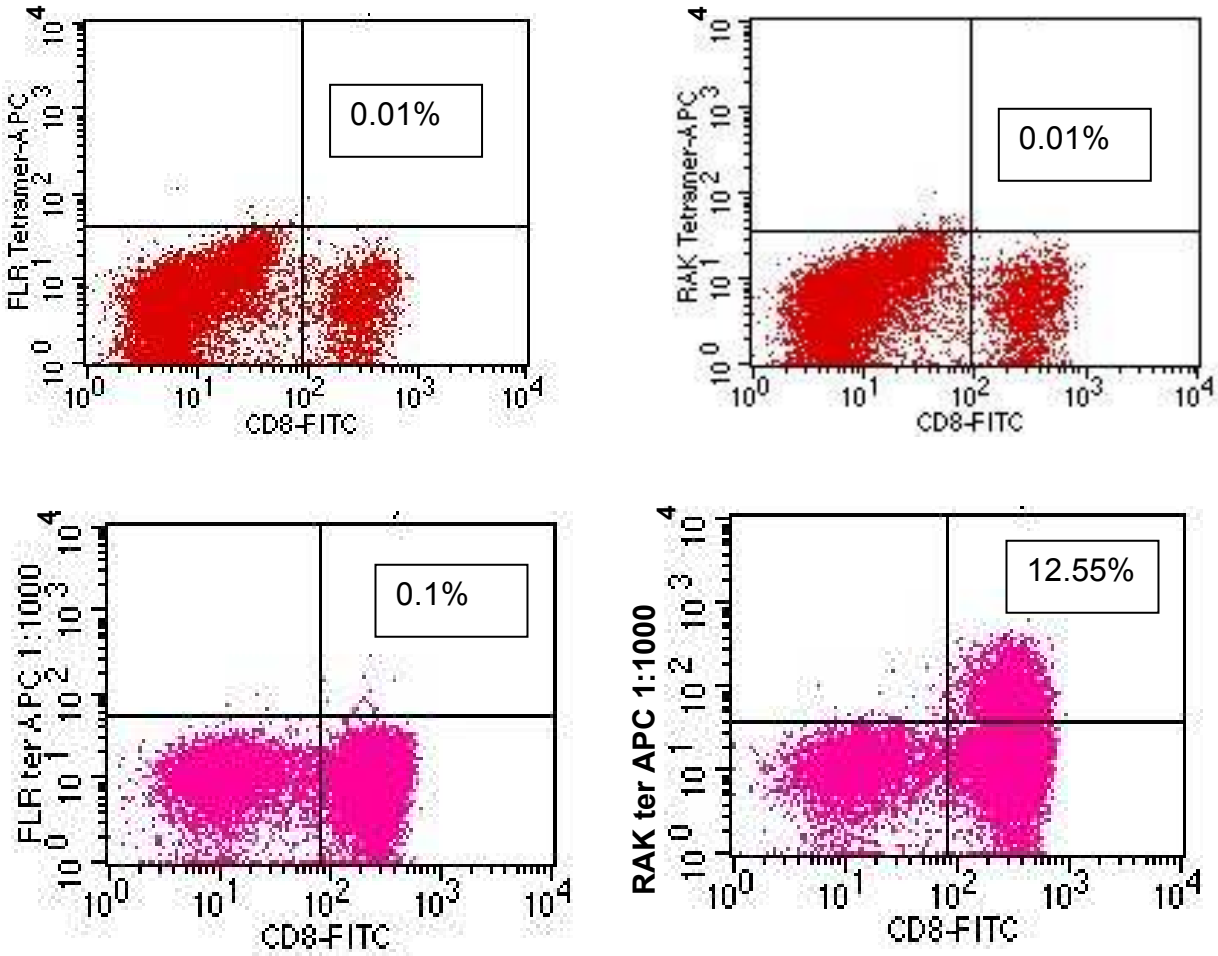
To test the efficacy of *in vitro* synthesized and purified EBV BZLF1 protein, human monocyte-derived DCs were generated after a 3 day culture in the presence of GM-CSF, IFN-α, and purified GST-BZLF1 protein. DCs that were treated in the same way, but cultured in the presence of BSA instead of GST-BZLF1, served as the control group. The BZLF1-loaded DCs were then co-cultured with human peripheral blood

leukocytes (hu-PBL) with the matched HLA type for two weeks. On day 14, the expansion and activation of BZLF1-specific CD8+ T cells was determined by flow cytometry (*figure 3*).

Figure 3

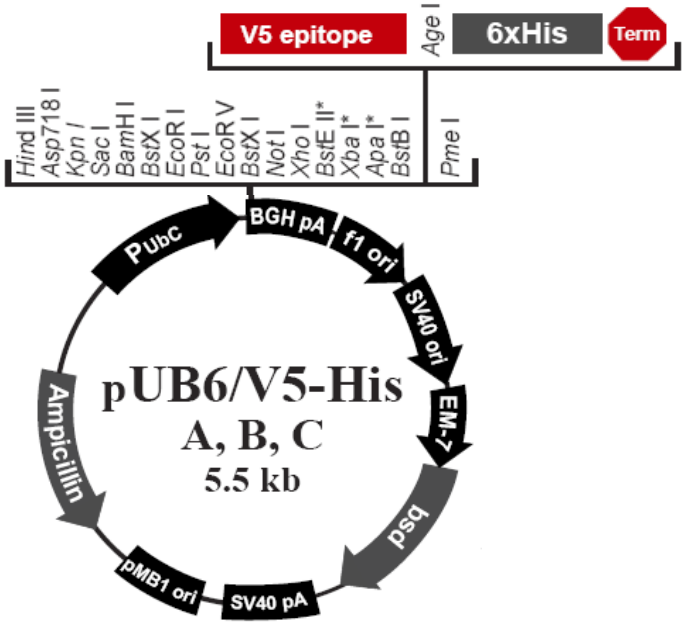
BZLF1-GST promotes RAK CD8+ specific T cell expansion

After cloning and purification, the function of this process was analyzed. The expansion of CD8+ CTLs was determined by flow cytometry. A tetramer system was used to detect antigen-specific CD8+ CTL expansion (RAK tetramer), while FLR tetramer was used as a negative control.
Top (red): cultured in the presence of BSA
Bottom (pink): cultured in the presence of GST-BZLF1



The intention of Aim 1-Goal B was to synthesize EBV BZLF1-V5-His fusion protein *in vitro* from mammalian cells. In order to generate a BZLF1 protein fused with the V5-His tag, EBV BZLF1 was amplified by PCR and cloned into a vector (pUB6/V5-His vector, *figure 4*). The pUB6/V5-His vector was prepared by digestion with restriction enzymes (BamHI and EcoR I) and dephosphorylation with calf intestinal alkaline phosphatase. The vector was then gel purified. The cloning was successful as evidenced by many more colonies produced from ligation in the presence of the insert compared with the negative control. The positive clones were identified by colony PCR and restriction digestion.

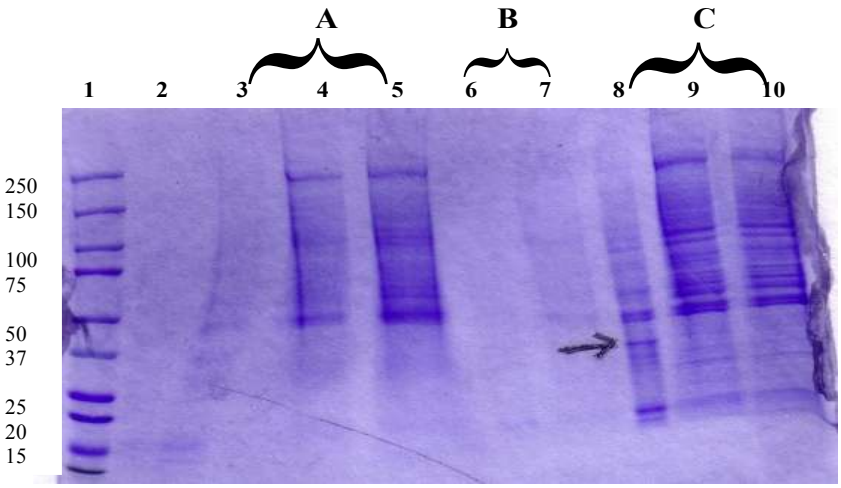
Figure 4



BZLF1 protein fused with the V5-His tag was cloned into a pUB6/V5-His vector (above).

Meanwhile, stable Chinese Hamster Ovarian (CHO) cell lines were generated. The culture was scaled up, the extract was prepared, and the cells were harvested by trypsinization. The BZLF1 protein was purified following standard protocol provided by Invitrogen's ProBond Purification System. The cell lysate was prepared under native conditions and added to a prepared His affinity column. The lysate was allowed to bind to the resin before the column was washed and the protein eluted. The protein concentration was measured at UV280 and the eluted fractions were analyzed by SDS-PAGE (*Figure 5*) and Western blot using a monoclonal antibody against BZLF1 (*Figure 6*).

Figure 5



Lane 1: protein standard
Lane 2: + ctrl, BZLF1 protein (no tag)
Lane 3: A lysate (2.5ul, total lysate: 8ml)
Lane 4: A #1 fraction (30ul/1ml in total)
Lane 5: A #2 fraction (30ul/1ml in total)
Lane 6: B lysate (2.5ul, total lysate: 8ml)
Lane 7: B #2 fraction (30ul/1ml total)

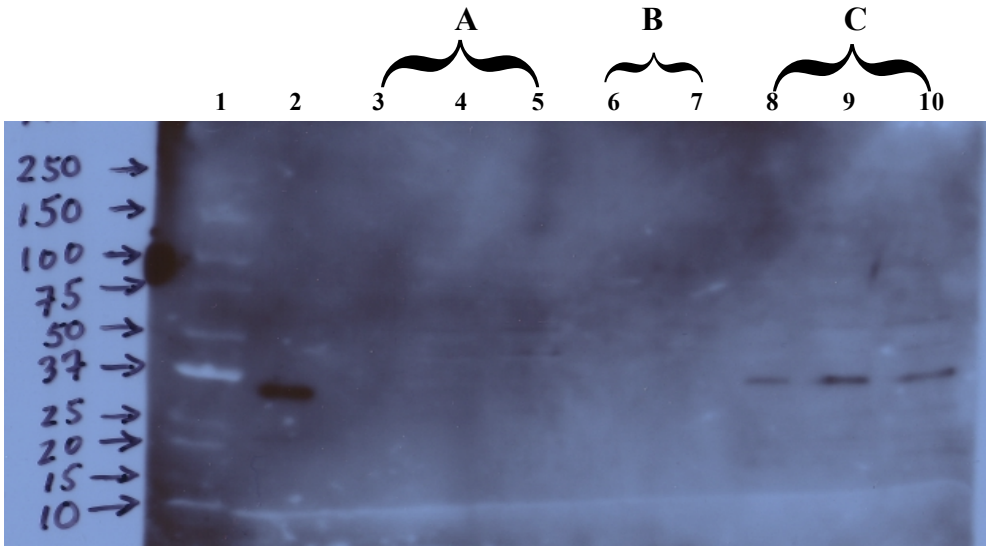
Lane 8: C lysate (2.5ul, total lysate: 8ml)
Lane 9: C #1 fraction (30ul/1ml in total)
Lane 10: C #2 fraction (30ul/1ml in total)

A: lysates from CHO/BZLF1 stable cells
B: lysates from CHO-S cells transfected w/ BZLF1 T72 hrs
C: lysates from CHO cells transfected w/ BZLF1 T72 hrs

1st antibody: anti-BZLF1 at 1:1000
2nd antibody: Goat anti-mouse 1:2500

SDS-PAGE analysis of BZLF1
A very faint band corresponding to 33-kd is seen. In addition, bands corresponding to co-purified cellular proteins are present.

Figure 6



Lane 1: protein standard
Lane 2: + ctrl, BZLF1 protein (no tag)
Lane 3: A lysate (2.5ul, total lysate: 8ml)
Lane 4: A #1 fraction (30ul/1ml in total)
Lane 5: A #2 fraction (30ul/1ml in total)
Lane 6: B lysate (2.5ul, total lysate: 8ml)
Lane 7: B #2 fraction (30ul/1ml total)

Lane 8: C lysate (2.5ul, total lysate: 8ml)
Lane 9: C #1 fraction (30ul/1ml in total)
Lane 10: C #2 fraction (30ul/1ml in total)

A: lysates from CHO/BZLF1 stable cells
B: lysates from CHO-S cells transfected w/ BZLF1 T72 hrs
C: lysates from CHO cells transfected w/ BZLF1 T72 hrs

1st antibody: anti-BZLF1 at 1:1000
2nd antibody: Goat anti-mouse 1:2500

Western Blot analysis of BZLF1
The Western Blot shows a specific band corresponding to 33-kd (lanes 8, 9, 10) similar to the band corresponding to the positive control (lane 2).

Discussion

For most healthy individuals, EBV infections are not life threatening and are generally effectively controlled by the immune system through the action of specific T lymphocytes [7, 8]. EBV-specific T lymphocytes recognize peptides from EBV proteins which are expressed on the surface of EBV- infected or EBV-transformed B lymphocytes [8]. Both CD8+ CTL and CD4+ helper T lymphocytes (HTL) can discriminate EBV-infected /transformed B cells and, therefore, are able to inhibit their growth [7]. Although most normal individuals are immune to EBV for their entire lives, the virus is not completely eliminated, but instead persists in a latent infection state [9, 10, 11], which is effectively controlled by the EBV-specific T lymphocytes [8]. However, in immunosuppressed individuals such as organ transplant patients, primary EBV infection usually results in Post-Transplant Lymphoproliferative Disease (PTLD), which often progresses into B cell lymphomas [12, 13, 14].

Unfortunately, current therapeutic approaches for PTLD and lymphomas are far from optimal. The reduction of immunosuppressive therapy allows for the immune-mediated viral elimination, but consequently, there is a high risk of organ rejection in these patients. In addition, the effectiveness of antiviral agents is not clear at this time [14]. One of the most effective ways to prevent/treat PTLD and B cell lymphomas is via adoptive immunotherapy using EBV-specific T lymphocytes [15, 16]. Unfortunately, this approach is labor intensive, costly, and not widely available [7]. The risk of PTLD and lymphoma increases significantly in patients who lacked immunity to EBV before transplantation and who are undergoing immunosuppressive therapy [17]. It is possible

that this risk could be lowered if EBV-seronegative patients could be immunized to stimulate their T lymphocytes, but unfortunately such a vaccine is not yet available [7].

PTLD is a devastating post transplantation complication and [18] as organ transplants continue to increase in number, and the risk of organ rejection decreases because of the improvements in immune suppressive therapy, PTLD will continue to be a significant problem [1]. Since physicians lack a reliable treatment of PTLD, it is important to develop a successful way of treating these patients as soon as possible.

An appealing and practical approach to develop vaccines that are intended to elicit antigen-specific T cell responses is the use of synthetic peptides representing CTL and HTL epitopes [7]. This strategy has been explored for various viral and malignant diseases [19, 20, 21, 22]. A large number of T cell epitopes derived from EBV latent and lytic cycle antigens have been identified, and many of these are being considered as potential vaccine candidates [23, 24]. Many of these efforts are focusing on using histocompatibility antigen (HLA) class I-restricted peptide epitopes to induce CD8+ CTL responses to EBV, since these cells are considered to be the prime effector cells that will presumably destroy the virus-infected and transformed cells [7]. There is also recent evidence that CD4+ T lymphocytes can function as potent effectors for inhibiting EBV-induced B cell proliferation, which would be the initial step of PTLD [25].

An important possible drawback of T cell peptide epitope vaccines is the limitation of HLA restriction. Most CTL and HTL peptide epitopes will only be useful in the limited proportion of individuals who express the appropriate HLA allele. However, in the case of HLA class II epitopes, some peptides have been found to bind indiscriminately to more than one HLA allele [26, 27, 28]. In some cases, these

indiscriminate epitopes can bind up to 10 common HLA alleles, indicating that the majority of the population would recognize these epitopes [29]. Therefore, a large proportion of patients would be able to benefit from a vaccine in which the peptide is able to bind to multiple HLA alleles.

We hypothesized that a vaccine of BZLF1 full protein transduced monocyte-derived dendritic cells (DCs) would stimulate the expansion of EBV BZLF1-specific cytotoxic CD8+ T lymphocytes (CTLs) efficiently. We demonstrated that this did, in fact, occur. When T-cell expansion was analyzed by flow cytometry, we determined that BZLF1-GST promoted RAK CD8+ specific T-cell expansion, while the BSA did not.

In order to determine the efficiency of this approach in humans, an *in vivo* study of the vaccine using severe combined immune deficient (SCID) mice will be done. In order for the T-cell expansion to occur effectively, the conditions under which the BZLF1 protein is purified must first be optimized, however. The SCID mice lack functional T and B lymphocytes. This lack of an effective immune response coupled with the intact hematopoietic microenvironment allows the SCID mouse to be reconstituted with human bone marrow or peripheral blood lymphocytes [30]. Giving these mice human blood from individual donors who are EBV positive gives rise to human B-cell tumors in the mice. We hypothesize that giving these mice the BZLF1 protein will increase RAK CD8+ T-cell expansion and prevent PTLD in these animals.

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